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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re of Application of:	**Berend JONGSMA et al.**		
Serial No.:	**09 / 775,750**	Group No.:	**1648**
Filed:	**February 2, 2001**	Examiner:	**S. Foley**
For:	**IN OVO PROTECTION AGAINST INFECTIOUS BRONCHITIS**		
Confirmation No.:	**9381**		
Customer No.:	**25291**		

Commissioner for Patents
Washington, DC 20231

Sir:

DECLARATION OF FRANS DAVELAAR UNDER 37 C.F.R. §1.132

1. I, Frans Gerrit Davelaar, of Harderwijkerstraat 85, 3881 EG Putten, The Netherlands, do declare and state as follows:

2. I qualified as a veterinary surgeon in 1972 at the State University of Utrecht, The Netherlands. I obtained my Ph.D. at the same university in 1981 in the field of veterinary science. In 1972, I became a municipal meat and hygiene official and a government meat inspector. In 1974, I jointed the State University of Utrecht, Department of Poultry Diseases, as a Senior Lecturer. While there, I was extensively involved in teaching and research, mainly in the field of viral diseases in poultry. My work at the University resulted in the thesis entitled: "Immunization of Young Chicks Against Infectious Bronchitis and the Role of the Harderian Gland in the Immune Response." I am also the author of 65 additional scientific publications in the field of veterinary science, mainly poultry disease. In 1985, I also became the Visiting Lecturer on Poultry Diseases at the University of Zimbabwe in Harare. In 1989, I was named head of the Department of Poultry Diseases at the State University of Utrecht. In 1992, I joined Solvay Duphar B.V. (from 1997 on, Fort Dodge Animal Health – now a division of Wyeth) as Clinical Research Manager Poultry. I am a member of the World Poultry

Science Association, the World Veterinary Poultry Association, and the International Committee on Avian Coronaviruses. I am also Chairman of the Certification Board for Veterinary Specialists in Poultry Diseases of the Royal Dutch Veterinary Association.

3. I am one of the named inventors in the above-captioned patent application. I have read and I believe I understand the specification and claims (as currently amended).

4. I am thoroughly familiar with Example 3 in the specification of the above-captioned application. All the tests described therein were either conducted directly by me, or were conducted under my close supervision.

5. Briefly described by way of summary, five groups of commercial eggs were obtained from the supplier Pronk, in Meppel, The Netherlands. Each group then received an *in ovo* dose of IB vaccine at one of the following EID_{50} levels: $10^{2.0}$, $10^{1.0}$ $10^{0.0}$, $10^{-1.0}$ and control (no vaccine). As reported in Example 3, the "% hatched" ranged from 86-93%. The "protection %" of the hatched chicks against challenge with virulent IB virus at 3 weeks of age ranged from 89-100% (Table 13). These results were obtained using the highly scientific and accepted CST ("cilla stopping test") methodology. CST is described in the paragraph bridging pages 11 and 12 of the specification.

6. I further state herein that the eggs utilized as part of the experiment for Example 3 all had maternal antibodies to IBV (as a result of post-hatch vaccination of the hens which bore them). This was confirmed by a progeny test of more than eight dozen sample chicks at day one of age. The results of HI ("haemagglutination inhibition") titration to IB M41 antigen (a Massachusetts strain) indicated a mean 2log HI titre within the range of 6.3 – 8.3. (The sample chicks were not utilized further as part of Experiment 3.)

7. I understand that the claims of the application have been rejected under 35 U.S.C. §103 as allegedly being obvious in view of the article by Wakenell et al. from the American Journal of Veterinary Research (vol. 47, pp. 933-938, 1986) entitled: "Chicken Embryonal Vaccination with Avian Infectious Bronchitis Virus". I have read and believe I understand the disclosure of this article.

8. Regarding Wakenell et al., TABLES 4 and 5 and the corresponding text appear to be the most salient. Described therein are the results of tests in which 18-day old chicken embryos were vaccinated with IB vaccine. It appears that the highest level of protection achieved by surviving chicks was 86% (in TABLE 4) against challenge with virulent IB virus (for the quantity of IB vaccine – "100 PFU" - which appears to correspond to that set forth and claimed in the present application).

9. I further note that the "86%" results in Wakenell et al. were based on "% Protection against signs of respiratory tract disease". These "signs" included individual clinical observations of "wheezing, gasping or coughing", as reported by the authors (see TABLE footnotes). Further details regarding these observations are apparently not included in the article. The authors do report another result of 63% in TABLE 5 for another experiment, using these same "signs" as one standard.

10. A further analysis was conducted by Wakenell et al. using tracheal isolation of C-IBV. The reported results are significantly worse overall, in terms of % protection against IBV. Results ranged from 50% protection in TABLE 4 to 68% in TABLE 5.

11. I also feel that the results reported in TABLES 4 and 5 would have been even worse if the chicks had been challenged at 3 weeks post-hatch, instead of 4 weeks. Their bodies would have been younger and weaker, with less time to develop the antibody resistance to stave off the IB onslaught.

12. As noted above, CST was utilized in the present application to assess protection against virulent IBV. This is well-regarded method which is at least as

exacting and reliable as either of the methods set forth in Wakenell et al. As confirmation, I have attached as "**Exhibit 1**" hereto an article entitled "Possibilities and Limitations of Combined Vaccines", from the Proceedings of the International Symposium on Infectious Bronchitis, June 23-26, 1988. This article is cited not for its substance, but for its testing methodologies. Tables 1 and 5 on pages 313 and 315, respectively, compare post-vaccination challenge results after inoculation with a combination IBV-NDV vaccine. Results are listed in terms of observed "clinical signs" (similarly to Wakenell et al.), as well as the "ciliostasis test". The ciliostasis test is simply another name for CST. As Tables 1 and 5 indicate, the results obtained using the two testing methodologies are highly comparable, and essentially mirror one another.

13. The results achieved by the present invention indicate that the vaccine and method described in the above-captioned application attain significantly better results than would have been expected following Wakenell et al. Wakenell's implication is that one could only have expected relatively mediocre results when an IB vaccine is administered to embryos bearing maternal antibodies. This would normally have made sense, since material antibodies have often been shown to interfere with a vaccine-generated immune response. The present invention has countered the conventional wisdom, however, and attained excellent vaccine protection in embryos with maternal antibodies.

14. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements my jeopardize the validity of the above-captioned application and any patent issuing thereon.

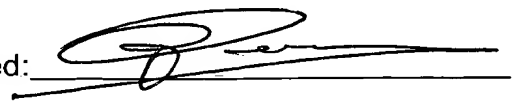
Signed:  Date: 27 January 2003

EXHIBIT 1 TO DECLARATION OF
FRANS DAVELAAR

Deutsche
Veterinärmedizinische
Gesellschaft e.V.
-Fachgruppe Geflügel-
and
World's Poultry
Science Association
European Group No. 7
"Hygiene and Pathology"

I. INTERNATIONAL SYMPOSIUM ON INFECTIOUS BRONCHITIS

PROCEEDINGS

RAUISCHHOLZHAUSEN, WEST-GERMANY,
JUNE 23 - 26, 1988

Intervet International B.V.

POSSIBILITIES AND LIMITATIONS OF COMBINED VACCINES

C. Schrier and D. Cornelissen

SUMMARY

The increasing number of vaccinations in modern poultry husbandry calls for the development of combined vaccines. Whereas this does not seem to cause large difficulties for inactivated vaccine combinations, the problem of interference is a major obstacle in combining live vaccines. Several trials were performed to establish the pathogenicity and immunogenicity of a newly developed IBV-NDV combination vaccine. For proper evaluation of the potency of the IBV component, different challenge evaluation tests were compared. Results indicated that ciliary acitivity, when examined at the right time after challenge, is a reliable and objective criterion for assessment of tracheal immunity. It was shown that with this IBV-NDV combination vaccine no apparent interference occured and that good protection against infection with pathogenic NDV and IBV (Massachusetts type) could be obtained.

INTRODUCTION

Modern intensive poultry production has increased the infection pressure for many diseases and therefore increased the need for vaccination programmes in those situations where eradication programmes are not feasible.
Because of the increasing number of vaccinations the development of effective combined vaccines is desirable.
The question whether different inactivated antigens can be combined is basically a pharmaceutical-technical one.
When presented in the right quantity and the right formula each component of the present available combined inactivated vaccines can induce immunity to the same extent as when presented separately. In that sense the limits of the immunocompetity of the chicken immune system have not yet been reached. Compatibility of different live vaccines can be more problematic. For such combination it is necessary to establish whether the components have no negative effect on each other. Especially virus vaccines against respiratory diseases tend to cause interference problems, mainly based on the fact that they aim for the same target cells. Conflicting data have been reported about the occurence of mutual interference when combining IBV and NDV (2, 3, 4, 5, 6 and 7).

Both the impact of vaccine titres and strain characteristics on the induction of immunity and the different methods used for the evaluation of the protective effects of such combined vaccines could be an explanation for these discrepancies in results.
It is of continuing interest to investigate whether the negative effects of a combined IBV-NDV vaccine can be overcome or at least reduced to acceptable levels by selecting strains which are mutually compatible. One strain with promising characteristics, both immunogenetically and compatibility wise, proved to be the spontaneously haemagglutinating IBV-Massachusetts strain Ma5. This strain was developed as a vaccine strain for one-day-old chickens and has shown in previous experiments to be safe and effective in chickens both with high and with low maternally derived antibodies.
When combining this strain with a Newcastle disease vaccine strain, good protection both against infection with a NDV challenge strain and infection with an IBV challenge strain (Massachusetts type), can be obtained.
This paper describes the results of vaccination and challenge experiments carried out with the Ma5 and the Ma5-NDV combination.

MATERIALS AND METHODS

Chickens
SPF chickens (white leghorn type) derived from our own breeder flock and broiler chickens obtained from a commercial supplier were used. The birds were kept in negative pressure isolators throughout the experiments.

Vaccines
Both commercially produced vaccines and experimentally prepared vaccines were used.

Challenge
IBV-challenge was performed by eye-drop administration of either the H41 U.S.D.A. challenge strain (U.S.D.A. IBV-LV-CEO serotype Mass 1841, lot no. IB 8011, U.S.D.A. APHIS NVSL) or the H41-challenge strain originally supplied by CVI, Weybridge (U.K.). The viruses were diluted in T.P.B. to achieve approximately 10^6 EID_{50}/ml and 0.1 ml per bird was administered. NDV-challenge was performed with the pathogenic NDV-Herts challenge strain and 10^6 EID_{50} per bird was administered intramuscularly.

Virus recovery
For virus recovery attempts the tracheas were removed aseptically and cut into pieces. The trachea cuts were suspended in 3 ml TPB (+ 1.5% gentamycine, 1000 ug/ml) and from each suspension 0.2 ml/egg was inoculated into at least six 9-11 days old embryonated SPF-eggs.
After further incubation for eight days, embryos were harvested and inspected for morphological changes. If more than 20% of the embryos were found positive for IBV, virus reisolation was scored positive. Birds from which IB-virus could be reisolated were regarded as unprotected against the challenge.

Ciliostasis test
Tracheas were collected in 5 ml medium (M199/P10). From each bird five tracheal rings were prepared and observed under a microscope for ciliary activity according to the method of Andrade et al. (1).

Immunofluorescence test
Cryostat sections of the tracheas were fixed in acetone for 10 minutes at room temperature. Following fixation, tissues were incubated for one hour at 37°C with a monoclonal antibody, MoAb 25.1, directed against the matrix protein (E_1) of IBV (kindly provided by Dr. G. Koch, CVI, Lelystad, the Netherlands). The tissues were finally stained for one hour at 37°C with RAM/FITC (Nordic), subsequently mounted in buffered glycerol and examined using incident ultra-violet illumination.

EXPERIMENTAL DESIGN AND RESULTS

Experiment 1
This experiment was conducted to compare the difference in clinical signs and ciliary activity at four and at seven days after an IBV-challenge in vaccinated and unvaccinated birds.
For this purpose one-day-old SPF-chickens were vaccinated by eye-drop with 10^4 EID_{50}/bird of the experimentally produced IBV vaccine strains A, B, C and Ma5. Five weeks post-vaccination the birds were submitted to a challenge with the M41 U.S.D.A. challenge strain.
Four and seven days post-challenge the birds were removed from the isolators, clinical signs were recorded and tracheas were examined for ciliary activity. As shown in table 1 the protection obtained with the different vaccine strains ranged from 60% to 100% as measured in the ciliostasis test four days post-challenge. When examined seven days post-challenge the ciliary activity has been completely recovered in all the vaccinated groups but not in the controls. These results indicate that for proper evaluation of the protective capacity of IBV vaccine strains the ciliostatis test should be performed four days post-challenge and not later. This is in agreement with the results of other investigations using different IBV challenge strains.

Experiment 2
To evaluate the assessment of protection in commercial broiler chickens vaccinated against avian infectious bronchitis (IBV) different challenge control methods were compared.
One-day-old commercial broiler chickens were vaccinated by eye-drop with a combined IBV-NDV vaccine: Ma5/Clone 30.
Four weeks post-vaccination the birds were submitted to a challenge with a M41-challenge strain (Weybridge). Four days post-challenge the birds were killed and tracheas were collected for histopathology, examination of the ciliary activity, virusreisolation, and for the indirect immunofluorescence test.
From the results (see table 2), it could be concluded that the Ma5/Clone 30 vaccine provided a good protection against a challenge with the M41 strain.
No significant differences were obtained between the results of the various challenge evaluation tests. In commercial broiler chickens virus reisolation post-challenge could be a problem due to the fact that agents other than IBV can influence the results by producing similarly embryonic lesions. It is most likely that the application of a specific IBV-antigen ELISA will solve this problem.

Table 1

Comparison results of ciliostasis test and clinical signs 4 and 7 days after challenge with an IBV-M41 challenge-strain

	% of birds with clinical signs post challenge		% of birds protected as measured in the ciliostasis test	
Vaccine	4 days p.c. (n=10)	7 days p.c.	4 days p.c. (n=5)	7 days p.c. (n=10)
Ma5	0	0 (n=13)	100	100
A	30	0 (n=13)	60	100
B	20	0 (n=14)	80	100
C	10	8 (n=12)	100	100
controls	90	67 (n=7)	0	0

- vaccination at one-day-old
- challenge with a M41-challenge strain five weeks p.v.

Table 2

Comparison IBV-challenge models

	Percentage of birds protected against a M41 challenge as measured by			
Vaccine	ciliostasis test 4 days p.c.	virusreisolation 4 days p.c.	histology 4 days p.c.	I.F.T. 4 days p.c.
Ma5/clone 30 eye-drop	97 % (n=33)	96 % (n=27)	100 % (n=14)	100 % (n=11)
none	0 % (n=26)	11 % (n=9)	0 % (n=11)	6 % (n=18)

		HI ^{2}log	VN ^{2}log
- vaccination at one-day-old			
- challenge with a M41-challenge strain 4 weeks p.v.	Titre at dD : M41	5.3 (0.7)	5.8 (1.4)

Table 3

Clinical symptoms post-vaccination

Vaccine	percentage of birds with clinical symptoms	
	4 days p.v. (n=6)	8 days p.v. (n=6)
H120 / clone 30 eye-drop	0	33
H120 / clone 30 spray	0	17
Ma5 / clone 30 eye-drop	0	17
Ma5 / clone 30 spray	0	0
none	0	0

- vaccination at one-day-old
- clinical symptoms (sneezing, gurgling) at 4 and 6 days p.v.

	HI ^{2}log
Titre at d0 : M41	7.6
NDV	5.2

Table 4

Ciliostasis test post-vaccination

Vaccine	Percentage of birds with ciliostasis	
	four days p.v. (n=6)	eight days p.v. (n=6)
H120 / clone 30 eye-drop	17 %	33 %
H120 / clone 30 spray	0 %	17 %
Ma5 / clone 30 eye-drop	0 %	0 %
Ma5 / clone 30 spray	0 %	17 %
none	0 %	0 %

- vaccination at one-day-old
- ciliostasis test at four and eight days post-vaccination

	HI ^{2}log
Titre at d0: M41	7.6
NDV	5.2

Table 5

Potency combined vaccine against IB

Vaccine	Four days post challenge with a M41-challenge strain	
	Percentage of birds with clinical symptoms post-challenge	Percentage of birds protected as measured in the ciliostasis test
H120 / clone 30 eye-drop	30 % (n=10)	58 % (n=12)
Ma5 / clone 30 eye-drop	0 % (n=10)	100 % (n=12)
none	80 % (n=10)	0 % (n=12)

- vaccination at one-day-old
- challenge with a M41 challenge strain five weeks p.v.

Table 6

Potency combined vaccine against ND

Vaccine	Challenge with NDV-challenge strain
	Percentage of birds protected
H120 / clone 30 spray	83 % (n=12)
Ma5 / clone 30 spray	100 % (n=12)
none	0 % (n=12)

- vaccination at one-day-old
- challenge with a NDV-challenge strain five weeks p.v.

Experiment 3
This experiment was conducted to compare two different IBV-NDV combination vaccines in inducing immunity against both an IBV and a NDV-challenge. One-day-old commercial broiler chickens were divided in five groups and placed in isolators. Two groups were vaccinated with the combination H_{120}/Clone 30 (one group by coarse spray, the other group by eye-drop method) and the other two groups were vaccinated with the combination Ma5/Clone 30 (one group by coarse spray, the other group by eye-drop method). One group remained as unvaccinated controls.
To evaluate the pathogenicity of the combined vaccines, six birds per group were removed from the isolators four and eight days post-vaccination. Clinical signs were recorded and ciliary activity in the tracheas was examined. Five weeks post-vaccination the birds vaccinated by eye-drop were submitted to an IBV-challenge and the birds vaccinated by spray were submitted to a NDV-challenge. Four days after the IBV challenge the birds were removed from the isolators, clinical signs were recorded and tracheas were examined for ciliary activity. From the birds receiving a NDV challenge clinical signs and mortality were recorded during 12 days p.c.
As shown in table 3 more vaccination reaction occured in the H_{120}/Clone 30 vaccinated group. This is in agreement with the results of the ciliostasis test (table 4). In the group vaccinated with the H_{120}/Clone 30 combination the protection against an IBV challenge was not sufficient. Thirty percent of the birds showed clinical signs post-challenge and 42 percent of the birds showed ciliostasis (table 5). The birds vaccinated with the Ma5/Clone 30 combination on the other hand, were completely protected against an IBV-challenge.
After the NDV-challenge (table 6) 2 out of 12 birds died during the observation period in the H_{120}/Clone 30 vaccinated group.
None of the birds in the Ma5/Clone 30 vaccinated group died or exhibited clinical signs during the observation period.

CONCLUSIONS

Although we have shown in our experiments that it is very well possible to overcome (to a great extent) the problems of interference when combining live vaccines, the exact mechanism is not understood right now.
Most of the mechanism of interference have been studied in in vitro cell culture system.
It is most likely that those mechanisms will turn out to be much more complicated or even totally different when studied in vivo.
From our experiments it is clear however, that when combining vaccine strains which are superior in their invasiveness and immunogenicity, interference problems do not occur or to a lesser extent.

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Intervet International B.V., Postbox 31, 5830 AA Boxmeer, the Netherlands